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ELECTRO-OPTICAL IMAGING OF F-ACTIN AND ENDOPLASMIC RETICULUM IN LIVING AND FIXED PLANT CELLS

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Abstract

Confocal and video micrographs of living and fixed alfalfa roots, onion epithelial and pear pollen cells illustrate the architecture of the cytoskeleton and endoplasmic reticulum in plant cells. Fixation of plant tissues to preserve cytoplasmic structure poses special problems. When possible, emphasis should be placed on the imaging of structures in stained living cells over time. The early events that occur when Nod factors or bacteria elicit nodule formation in alfalfa roots will illustrate several approaches to plant cell fixation, staining and imaging. The first observable events after Nod factor stimulation occur in root hairs and are changes in rates of cytoplasmic streaming, nuclear movements, and changes in the shape of the vacuole. Within ten minutes, the endoplasmic reticulum shifts position towards the tip of the root hair. For comparison, the endoplasmic reticulum localization in pollen tubes and onion epithelial cells will be illustrated. The actin cytoskeleton undergoes a series of changes over a twelve hour period. These changes in the cytoskeleton are spatially and temporally correlated with the observed growth changes of the root hairs. This dynamic change of the actin filament and endoplasmic reticulum and associated secretory vesicles in these root hairs suggests a mechanism for the observed root hair growth changes.

Key Words: actin, endoplasmic reticulum, alfalfa, video microscopy, onion epithelial cells, pollen tubes, cytoskeleton, Nod factors.

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Introduction

Video microscopy and computer manipulations have permitted better visualization of dynamic changes in the internal structures of plant cells [2] in both normal cells and in cells stimulated by internal or external agents. Differential interference contrast (DIC) light microscopy methods give an overall impression of the activities and structures in a living cell, but to insure the best identification of specific structures, fluorescently labeled antibodies or other specific tags are used [8]. Some labels easily enter living plant cells, but for other probes and antibodies the cells must be fixed. It is generally agreed that visualization and fixation can be more problematic in plant cells than in animal cells. Contributing to these problems are the presence of cell walls with strongly autofluorescent lignin, large often highly acidic vacuoles as well as autofluorescent chloroplasts. Dyes, such as the Ca^{2+} indicator fura-2, will quickly enter the vacuole of the plant cell, a structure with a high Ca^{2+} content. Hence the desired Ca^{2+} measurements in the cytoplasm will be obscured by vacuolar fluorescence. Vacuolar membrane disruption can occur during fixation resulting in the release of compounds disruptive to the cytoplasm and the cytoskeleton. It is important to select plant tissues with low autofluorescence as has been done here.

Plants are stationary and they respond to the environment by changing their growth. Early responses by plant cells leading to growth changes would appear to be linked to changes in the localization of actin filaments, microtubules, and the endoplasmic reticulum (ER) [9]. In order to detect such changes in the cell architecture we and others have tried to improve methods of staining and fixation of plant cells. Some of these will be described and their usefulness will be briefly discussed.

The plant cytoskeleton consists of actin, microtubules, and intermediate filaments and the actin filaments often colocalize with the endoplasmic reticulum [2, 12]. In animal cells the distribution of actin filaments is well known for fixed and living cells through the use of electron and fluorescence microscopy. Actin fila-

ments have been imaged in a number of plant cells with particular success in cryofixed specimens [11, 12, 18]. The method of tip growth and its relation to the actin cytoskeleton is of interest to plant biologists and a controversy exists regarding the presence or absence of actin at the tip of growing cells of germinating pollen and root hairs. Few actin-associated proteins (myosins, profilin), which might effect the actin arrays, have been characterized in plant cells. So far this field of study lags far behind what can be described for animal cells [6].

We have been particularly interested in the imaging over time of growing alfalfa root hairs after they have been challenged by the alfalfa specific factor NodRm-IV(S) [3, 5 and M. Bennett, D. Erhardt, S. Long and N. Allen, in preparation] in order to understand parts of the signal transduction pathway from the reception of Nod factors by the plant ending in the observed growth change. Nod factors produced by rhizobia are lipo-chitooligosaccharides with different long-chain fatty acids at the non-reducing glucosamine moiety [10, 14, 26]. Nod factors, such as NodRm-IV(s), specific to *Medicago sativa* (alfalfa) are produced by the soil bacteria *Rhizobium meliloti* in response to plant flavonoids [13]. These factors can cause many developmental changes in the plant eventually leading to the formation of pseudonodules on the roots [7]. Nod factors have recently been postulated to be plant growth regulators in that they can redirect plant growth in non-leguminous plants [19]. One of the first observed morphological changes is the deformation of growing root hairs and previous work implicated a role for the cytoskeleton in this change in growth [7, 18]. Hairs arise by tip growth from epidermal cells on the root (Fig. 1) and are long tubular projections often containing the cell nucleus. Within 3-4 hours after roots are exposed to *Rhizobia* or Nod factors, they will form a characteristic curl or show other deformities. Our aim was to image changes in the location of actin filaments and the endoplasmic reticulum in Nod factor challenged root hairs over time to further understand the basis for the growth form change.

The location of the ER in growing pollen tube tips was imaged in order to compare the location of the ER in tip growing root hairs and pollen tubes. The ER location obtained in living onion cells using a cooled charged coupling device (CCD) camera coupled with image enhancement illustrates the increase in resolution that can be obtained with this imaging mode in optically favorable specimens.

Materials and Methods

Plant material

Alfalfa seeds, *Medicago sativa* L., (AS13, Ferry-Morse Seed, Modesto, CA) were surface sterilized by soaking for 30 min in 70% ethanol followed by 100% bleach for 30 min. Seeds were rinsed thoroughly in distilled water and germinated on Whatman No. 4 filter paper moistened with distilled water in an inverted Petri plate at room temperature. Seedlings (24 hours old) were transferred to the surface of square Petri plates containing buffered nodulation medium (BNM) and 13% Bacto agar (Difco Laboratories, Detroit, MI). The BNM was composed of 2 mM $CaSO_4.2H_2O$, 2 mM 2-(N-morpholino)ethane sulfonic acid (MES), 0.5 mM $MgSO_4.7H_2O$, and 0.5 mM KH_2PO_4, with the minor salts 50 nM $Na_2Mo_4.2H_2O$, 50 nM H_3BO_3, 50 nM $FeSO_4.7H_2O$, 50 nM $MnSO_4.H_2O$, 16 nM $ZnSO_4.7H_2O$, 1 nM $Na_2MoO_4.2H_2O$, 0.1 nM $CoCl_26H_2O$, and 0.1 nM $CuSO_4.5H_2O$. The pH was adjusted to 6.0 using 2N KOH. The plates were sealed with Parafilm, and the seedlings were grown in the dark at 16°C. The inner onion epidermal peels were prepared as described in [2]. Pear pollen tubes were germinated on the microscope slide in pollen germination medium (PGM) [25].

Nod factor treatment

Tissues were treated with or without 10^{-8} M NodRm-IV(S) (obtained from Drs S.R. Long and D.W. Ehrhardt, Department of Biological Sciences, Stanford University, Stanford, CA) for time periods of 5, 10, and 15 min, and between 1 and 12 hours. Agar plates, containing the growing seedlings were placed horizontally in the growth chamber and 10^{-8} M Nod factor or buffer was applied along the root using a transfer pipette. The plated seedlings were kept horizontal, which ensured that the Nod factor remained in contact with the tissue for the duration of the treatment.

Fixation and staining

Actin. The lower 3 cm of treated and untreated alfalfa roots, which include both the young root hairs (48-72 hours old) and the zone of emerging root hairs (Fig. 1) were excised and placed individually on microscope slides containing 0.1 mM m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (Pierce, Rockford IL) [22] in actin stabilizing buffer (ASB) for 30 min. ASB was a modified version of the cytoskeletal stabilization buffer described in Abe and Davies [1] consisting of 5 mM 4 (-2- Hydroxyethyl)-1-Piperazineethanesulfonic Acid (HEPES), 10 mM Mg acetate, 2 mM Ethylene Glycol Bis-(b-Aminoethyl Ether) N,N,N',N'-Tetraacetic Acid (EGTA), and 1 mM Phenylmethyl-Sulfonyl Fluoride (PMSF) (Sigma, St. Louis, MO), adjusted to a pH of 7.5 with 1N KOH.

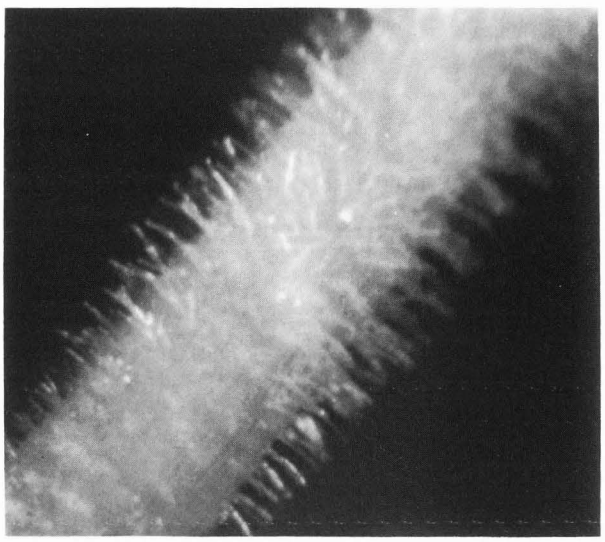

Figure 1. Micrograph of a root of *Medicago sativa* (alfalfa) showing root hairs emerging from the epidermal cells. The nod receptive hairs used in this study varied in length from 120-150 μm long and 10-22 μm wide. Field width = 550 μm.

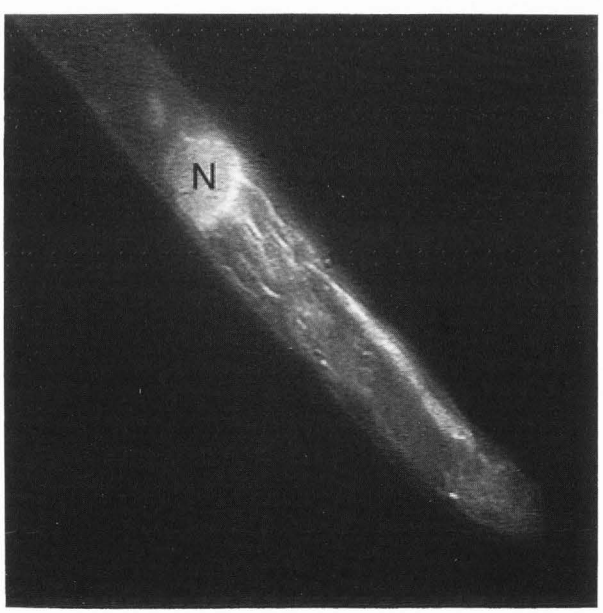

Figure 2. Alfalfa root hair (18 μm in diameter) stained for actin with rhodamine phalloidin. The nucleus is surrounded by actin filaments with one filament bundle extending from the nucleus. Figure 3F is an optical section taken a few microns below the section depicted in this micrograph.

After primary protein crosslinking with MBS, No. 1 coverslips were carefully placed over the root tips. By using filter paper as wicks, the tissue was perfused with freshly prepared 2.5% paraformaldehyde (Trioxymethylene) (Purified) (Fisher, Fair Lawn, NJ) and 0.05% glutaraldehyde (Ted Pella, Inc., Redding, CA) in ASB. The roots were fixed for 45 min in the dark. After the root tips were washed with ASB, they were digested for 30 min with 1.0% Cellulase (Sigma) followed by a 30 min extraction in 0.5% Triton X-100 in ASB. The root tips were briefly rinsed with ASB prior to staining.

The stabilized actin cytoskeleton was labeled by perfusing 100 μl of 0.17 μM rhodamine phalloidin (Molecular Probes, Eugene, OR) in ASB. Cells were placed in a moistened chamber for 4-6 hours in the dark and then rinsed. The root tips were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA) and gently crushed between microscope slide and coverslip and sealed with fingernail polish.

Endoplasmic reticulum. A stock solution containing 1 mg 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] in 1 ml dimethylsulfoxide (DMSO) (Molecular Probes) was diluted 1000:1 in BNM or PGM. Onion or alfalfa specimens were incubated in BNM stain solution for 10 min, then rinsed thoroughly three times in BNM and mounted for observation [20, 21]. Pollen grains were placed on microscope slides in PGM solution containing the $DiOC_6(3)$ stock solution in a 1000:1 dilution and germinated and recorded over time on the confocal microscope.

Microscopy

The alfalfa root hairs stained with rhodamine phalloidin were viewed on an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence and AVEC-DIC optics [4]. Most observations were made with a 63 x, 1.4 N.A. planapochromatic objective and further magnified with a 1.25 x zoom lens. The root hairs were found to have very little autofluorescence. Fluorescent images were visualized with a Hamamatsu Silicon Intensified Target camera (Hamamatsu Photonic Systems, Division of Hamamatsu Corp., Bridgewater, NJ) and then computer enhanced using the Image-1 system (Universal Imaging, West Chester, PA). The images were then recorded on a Panasonic TQ2028F optical memory disk recorder (OMDR), digitized, stored as TIFF files and later printed on Ep-2 photographic paper at 312 pixels per inch with a PowerMacintosh 8100 with Adobe Photoshop and Fuji or Codonics (Middleburg Heights, OH) Color printer.

The alfalfa root hairs and pear pollen grains stained with $DiOC_6(3)$ were imaged with a Biorad (Hercules, CA) 600 confocal laser scanning on a Zeiss IM35

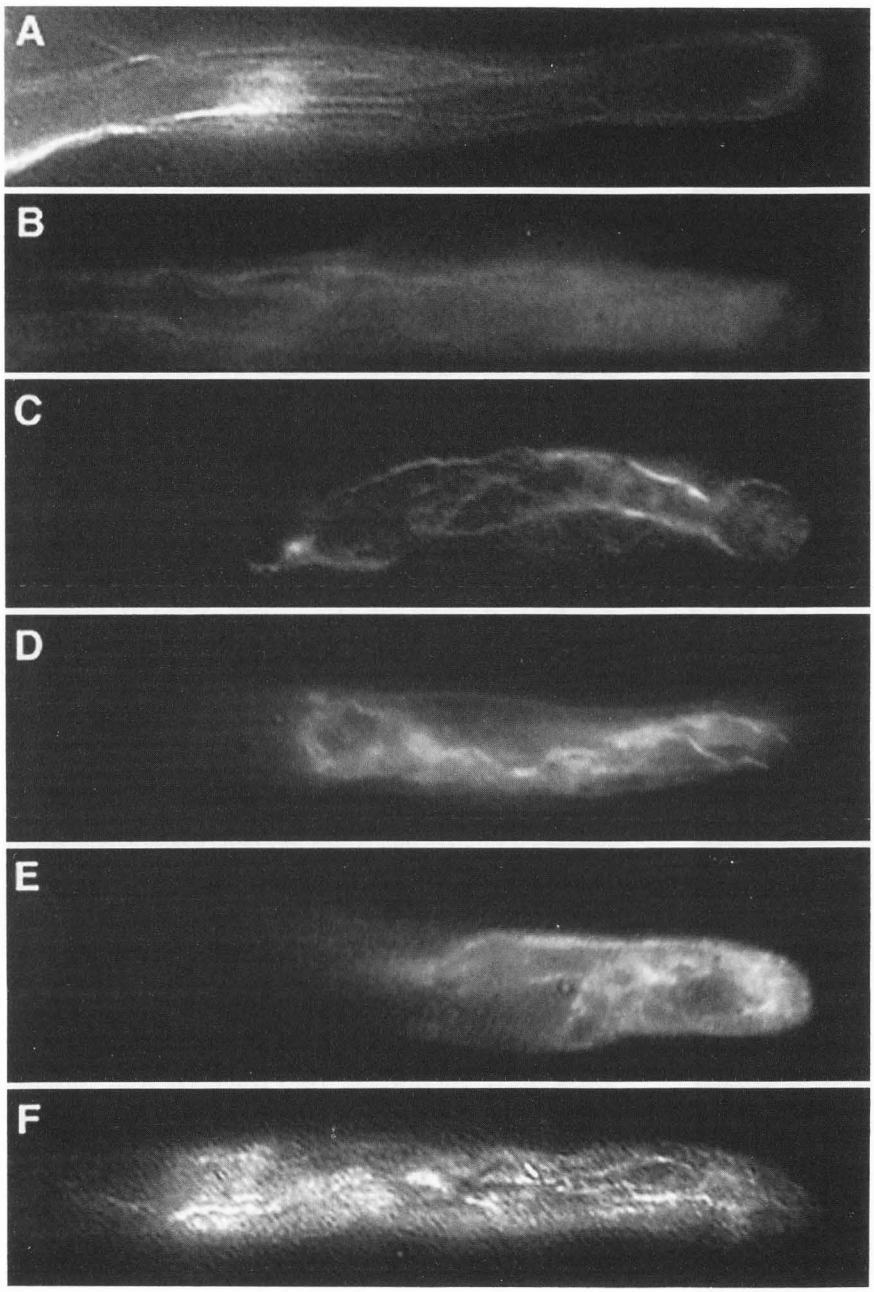

Figure 3. A to F. A montage of representative actin root hairs with rhodamine phalloidin stained actin filaments. Note that some hairs contained actin staining in the very tip. Long filaments of actin ran the length of the hairs and often connected into a more diffuse network of actin at the tip. When focusing through the tip, the network appeared to be connected to the long bundles entering the dome area.

microscope. The fluorescent images are confocal and the DIC images were recorded simultaneously, but they are not confocally derived. These images were time-lapsed on the OMDR, and some were digitized and stored on Image I as TIFF files. The onion epithelial cell was imaged on the Zeiss Axiophot equipped with a 63x, 1.4 N.A. objective and a matching oiled 1.4 N.A. condenser and a Hamamatsu Cooled CCD camera. A 4x zoom lens was present between the microscope and camera. The image was further enhanced and digitized using the Metamorph Image Analysis System (Universal Imaging). The TIFF files were then processed with the Adobe Photoshop and printed using a Codonics Color printer as above.

Results

Actin localization in alfalfa root hairs

25 seedlings with many root hairs were fixed, stained with rhodamine phalloidin, observed and recorded as described in Materials and Methods. The transparent hairs ranged in diameter from 10-22 μm and extended out in all directions from the thick, opaque root making imaging difficult. Representative images of such

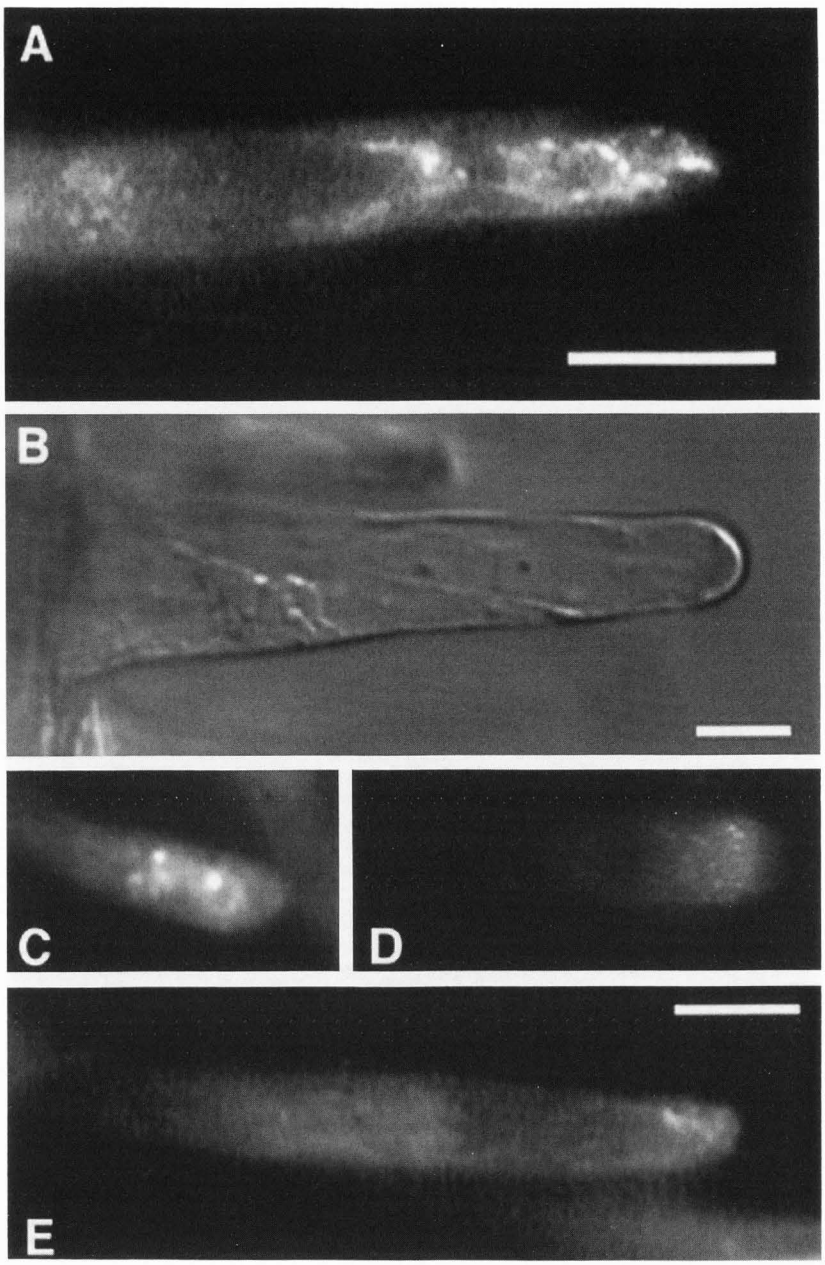

Figure 4. Changes in the actin cytoskeleton 10 min after exposure to 10^{-8} M NodRm-IV(S). The effectiveness of the fixation process was demonstrated by the fact that the appearance of the fixed hair imaged in DIC microscopy (B) did not change from that of living cells. In micrographs A, C, D, and E discrete foci of actin staining occurred in the tips of the root hair. The staining pattern indicated actin filament fragmentation. This striking pattern was observed only in cells exposed to Nod factor and was not present in older root hairs (those greater than 200 μm in length) or epidermal cells. The observed cytoskeletal alterations occurred prior to any observable changes in cell morphology. Bar = 20 μm.

stained and fixed root hairs can be seen in Fig. 2 and 3. Fig. 2 is a representative sample in a root hair of the intense actin staining that always surrounded the nucleus, whether it was present in the root hair or in the cell body (not shown). Actin filaments also course in linear arrays parallel to the long axis of the hair. Fig. 3 depicts 6 different root hairs in which the parallel actin bundles are best observed in A, B, C and E in the area of the cytoplasm where the microscope was in focus just below the plasma membrane and above the vacuole. No actin was seen in the vacuole. In some hairs a distinct cap of actin staining is clearly visible in the apical dome of the root hair as in A, C, E, and Fig. 2. Similar patterns of actin distribution have recently been described for *Hydrocharis* root hairs [21].

Actin distribution in NodRm-IV(S) stimulated root hairs

25 seedlings were exposed to 10^{-8} M NodRm-IV(S) for 10 min and Fig. 4 illustrates the dramatic shift in the rhodamine phalloidin staining pattern when compared to untreated root hairs. As seen in A, C, D, and E, a punctate staining pattern appeared at and near the tip of the root hairs. This pattern could be observed in 25% of

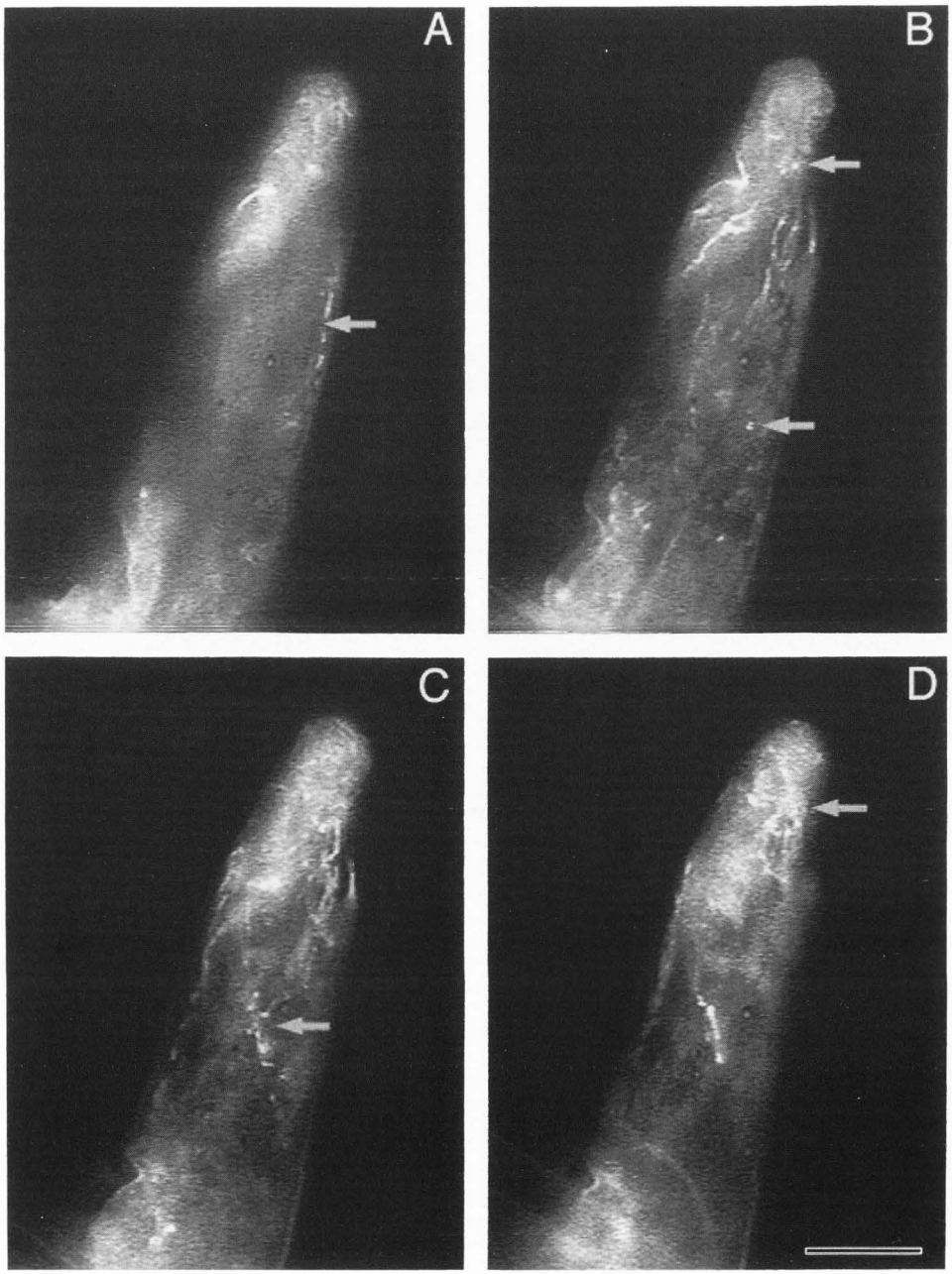

Figure 5. The effects of Nod factor on the cytoskeleton 15 min post-perfusion. This series of optical sections demonstrated the initiation of actin cable fragmentation marked by discontinuities in the longitudinal cables (arrow A, C) and the formation of discrete foci staining throughout the cytoplasm (arrow B, C). Although some actin filaments appear to remain, these micrographs show a widely occurring breakdown of the actin cytoskeleton. Bar = 20 μm.

all root hairs demonstrating actin labeling and was indicative of actin fragmentation. A qualitative assessment would indicate a somewhat higher diffuse actin staining than what was seen in unstimulated root hairs (Fig. 3). Fig. 4B is a DIC image of a root hair demonstrating that the cytoplasm of fixed and stained root hairs appeared very much like that of living hairs, thus indicating that very little apparent damage occurred during the preparation process. Fig. 5 is a series of four optical sections taken through a root hair 15 min post Nod factor perfusion. Fig. 5A presents the view just beneath the plasma membrane with the arrow indicating a fragmenting actin filament. Fig. 5B is an image taken with the microscope focused further into the cell demonstrating that actin foci were present (arrows). "Curled" actin filaments were seen near the left tip of the root hair. Fig. 5C again shows actin foci at the arrow. These images would indicate a widespread breakdown of the actin filaments into shorter fragments that may not be tightly stretched.

Although no images are shown for root hairs one hour after Nod factor exposure, we did find that the actin staining appeared normal and in DIC we could observe no differences between treated and untreated

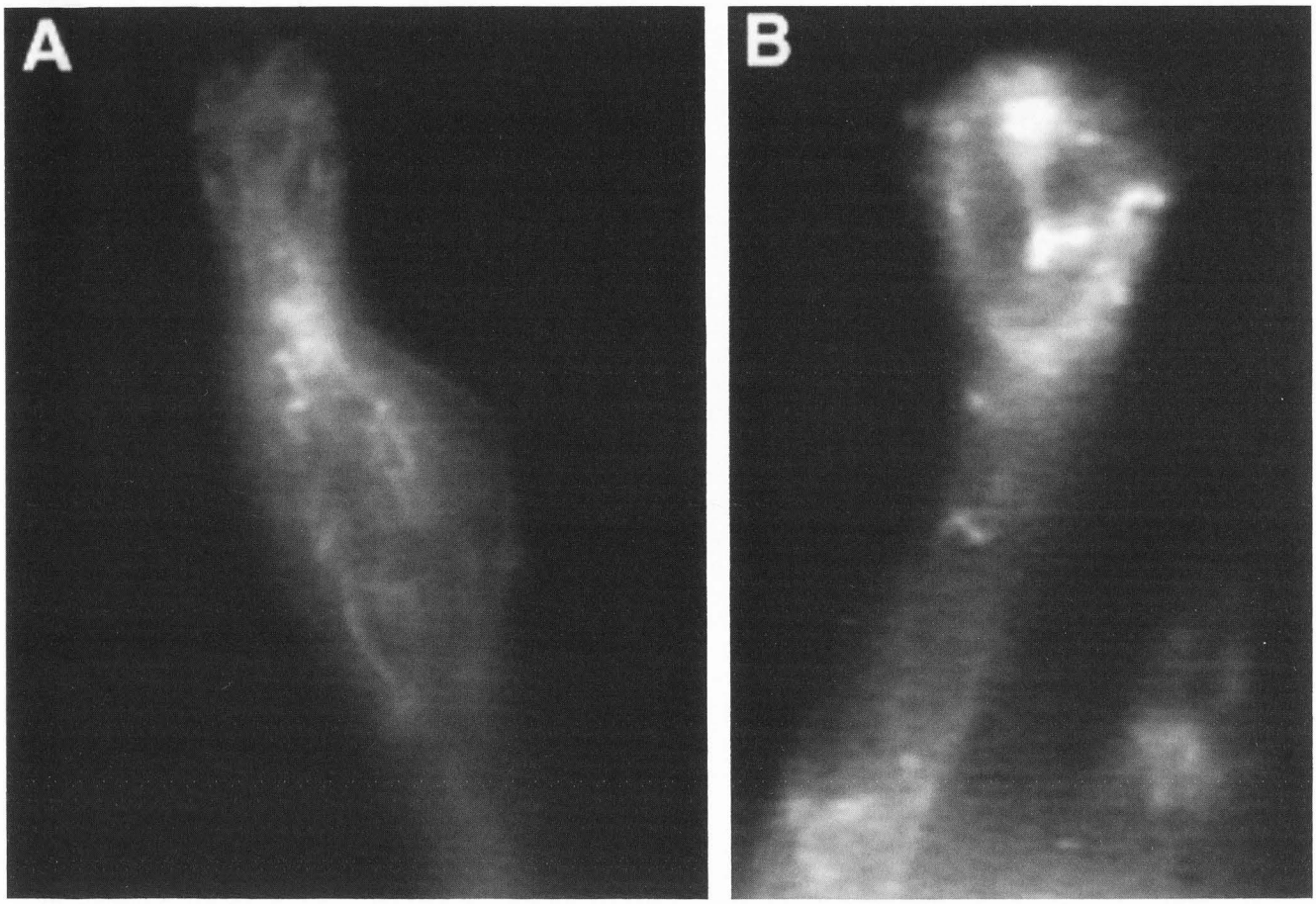

Figure 6. Rhodamine phalloidin labeled root hairs 3 hours post-perfusion with Nod factor with clearly visible root hair deformations. The root hairs were no longer cylindrical and became crooked or bulbous with obvious distortion. Within the tips of these structures, intense foci of actin staining were present very close to the plasma membrane. Within the same optical plane of these foci, a definite anastomosing pattern of filaments was often detected (A).

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hairs. Both return to a normal streaming pattern. However, 3 hours after Nod factor exposure and just when root hair deformations were visible, the actin localized into foci and very few actin cables were present (Fig. 6 A,B). Fig. 6A is a root hair of the type that shows a bulge below the root tip with very distinct actin foci. Fig. 6B represents a common deformation in which the root hair will branch. The actin foci seem to occur in an upside down horseshoe shape and no filaments were observed. The staining was only seen when the microscope was focused near the plasma membrane (the magnification is high and the optical sections were estimated to be ca. 350 nm) and suggests that in areas where the root hairs deform, there could be focal aggregations of actin filaments. The exact time course and length of time during which the actin filaments break down is not clear. Observations (not shown here) of root hairs exposed to Nod factors ten hours earlier revealed the normal patterns of long parallel filaments, nuclear staining, and apical dome staining. It has been possible to develop a method that has allowed the imaging of actin staining in Nod factor perfused root hairs over time and to gain some insight into the changing actin distribution in such cells.

Detection of the endoplasmic reticulum in normal and nod factor stimulated alfalfa root hairs

$DiOC_6(3)$ is a lipid stain that fairly selectively visualizes the endoplasmic reticulum [16, 17, 20, 23] in both living animal and plant cells. Alfalfa root hairs were allowed to take up the dye for 10 min, placed in a perfusion chamber in BNM, and recorded at a rate of 1 frame every 4 seconds on the OMDR. Fig. 7a is a dual image video micrograph of the same root hair imaged in DIC and fluorescence microscopy modes. A large vacuole can be seen with no dye content. The unstimulated cells were observed and recorded over 30 min and

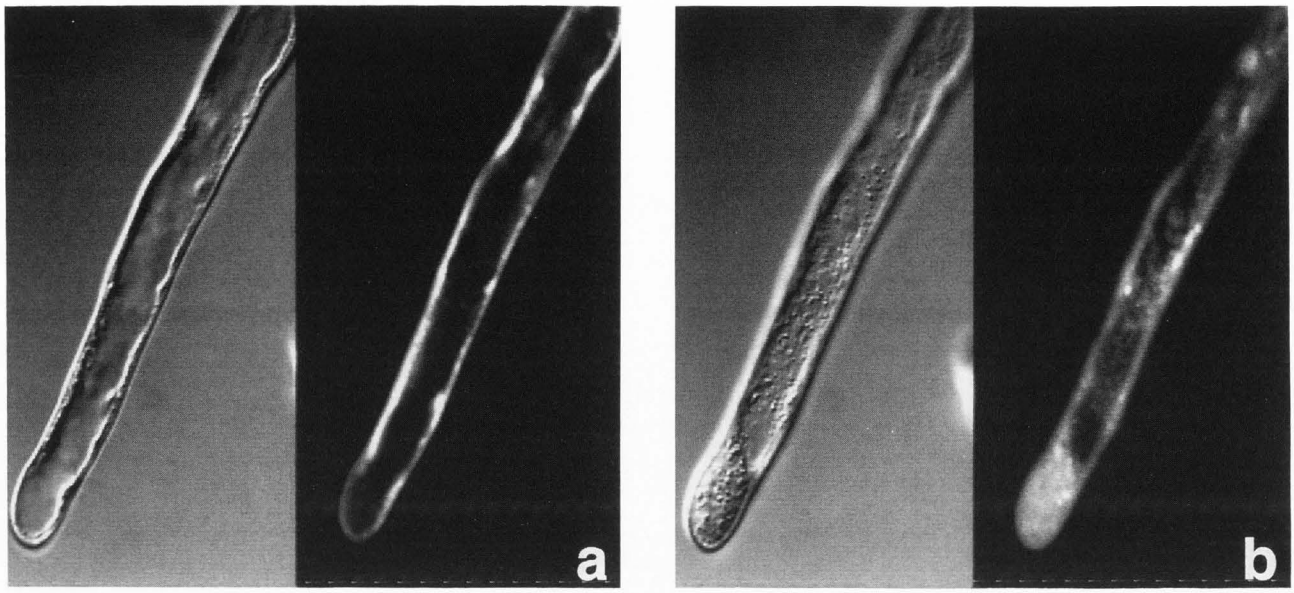

Figure 7. Confocal laser scanning micrograph of an alfalfa root hair stained with $DiOC_6(3)$ specific for the ER. Figure 7a depicts the unstimulated hair in DIC and fluorescence mode. The vacuole fills most of the root hair tip and the cytoplasm show general staining for ER. After NodRm-IV(S) stimulation, the cytoplasm rich in ER staining migrates to the tip of the root hair displacing the vacuole. The diameter of the root hair was 18 μm.

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at this point the Nod factor was perfused across the root. Fig. 7b was recorded 12 min after Nod factor perfusion. The vacuole had shifted position as compared to Fig. 7a and many vesicles (DIC) as well as the stained ER had shifted towards the tip of the root hair. The time frame of this shift correlated well with the observed changes in the actin architecture after Nod factor perfusion.

Imaging of the endoplasmic reticulum in pear pollen tubes

Pollen tube and root hair tip growth may be based on the same phenomena [9] and it is felt that both actin and the ER are involved in this growth extension. The same staining methods were employed to image the ER in pollen tubes as was described for the alfalfa root hairs. Dual, simultaneous images (DIC and confocal fluorescence) of the tip and lower end of a germinating pollen tube from an hour-long OMDR recording of the germination of the pollen grain and growth of the pollen tube are seen in Figure 8. In that video recording, the cytoplasm in DIC was seen to stream vigorously carrying many small vesicles and particles to the tip of the pollen tube and the ER also was shifting position rapidly over time. The ER was present throughout the cell. The DIC image demonstrated that the very tip of the pollen tube was filled with cytoplasm, but the fluorescently labeled ER was excluded from that very tip. This differed from the alfalfa root, in which the ER occupied the very tip of the root hair.

Imaging of the endoplasmic reticulum in onion epithelial cells

The first and most commonly used plant cell for visualization of the ER has been the onion epithelial cell [2, 16] . These cells are optically favorable since they are present in very thin, transparent sheets and their cytoplasm is less dense than that found in most plant cells and in alfalfa root hairs or pollen tubes. It is possible to distinguish two different types of ER in onion cells [2]. Fig. 9 depicts a small area of an onion cell in focus just below the cell wall and plasma membrane. The cells were stained with $DiOC_6(3)$ and imaged using the Hamamatsu Cooled CCD followed by computer-enhancement with the Metamorph image analysis system. Staining made it possible to see the typical network of ER tubules, the saccules and a clump of putative secretory vesicles. This image would indicate that the dye is quite specific for the ER. We have not yet obtained such images from alfalfa root cells.

Conclusions

It is now possible to image with greater clarity some of the cytoskeletal elements present in plant cells [22] including now alfalfa and *Hydrocharis* [21]. This will lead to further understanding of many processes involving the cytoskeletal architecture. The shape of a plant cell is in part due to the underlying cytoskeleton [15, 27] and it is known that actin plays a significant role in tip

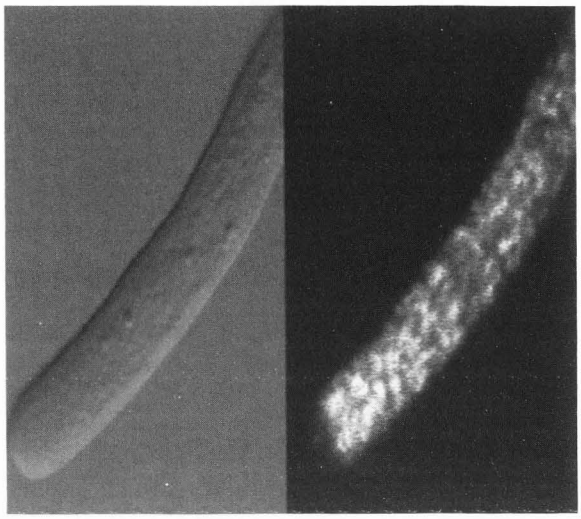

Figure 8. Confocal laser scanning micrograph of a pollen tube germinating from a pear pollen grain and vitally stained with $DiOC_6(3)$. Note that the ER staining does not extend to the very tip of the pear pollen tube. This is in contrast to the staining pattern observed for ER in alfalfa root tips.

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growth processes in certain cells as well as in cellular elongation in general [9]. Hence if there are changes in the cytoskeleton during the normal growth process, one might expect to see changes in form. We have demonstrated that there is a breakdown of the actin cytoskeleton after exposure to NodRm-IV(S), which is correlated in time with the observed growth change (root curling) at three to four hours. We also show that the changes in distribution of the actin and ER in response to Nod factors occurred within ten minutes after exposure and resulted in a fragmentation of actin filaments and a movement of the ER and/or ER derived vesicles towards the root hair tip. It is not clear if the actin filaments are acting as tracks for vesicle movements to discrete locations or if the cytoskeleton itself serves as a template for morphogenesis. In either case, it is reasonable to predict that a change in the cytoskeleton would result in a change in the shape of the root hair.

The dome of the alfalfa root hair tip contains a meshwork of actin filaments, which has also been seen in *Hydrocharis* root hairs [21]. Ridge [18] using a different preparation method does not see actin at the tip of alfalfa root hairs. It will be important in the future to label the actin filaments in living alfalfa roots and to observe the precise actin localization at the tip of the root hairs and the general change in actin localization over time.

There is a difference in staining of the ER of pollen tube tips and alfalfa root hairs, which may indicate that

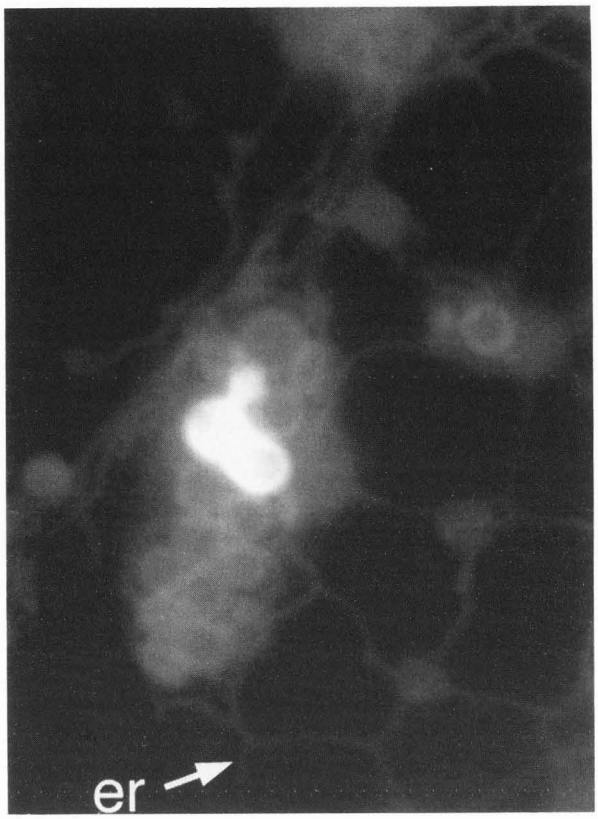

Figure 9. Onion epithelial cells from the adaxial side of an onion bulb scale stained with $DiOC_6(3)$. The ER tubules form a polygonal network (arrow) tightly appressed to the plasma membrane. A cluster of putative secretory vesicles are seen near the center of the image. Saccules are present where the tubules join each other. Field width = 24 μm.

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the deposition of ER derived vesicles may differ in these two types of cells and hence tip growth itself may differ in some ways in these two cell types.

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Discussion with Reviewers

E. Ralston: From the experiments described in the paper it is clear that actin filaments and ER distribution

are modified after stimulation by the Nod factor, but it is not possible to access how critical these changes are. Have you tried to depolymerize the actin filaments before applying the Nod factor? What happens to the microtubules?

M. Terasaki: Does cytochalasin block the accumulation of the endoplasmic reticulum at root tips after application of the NOD factor?

Authors: If cytochalasin is added, tip growth stops, so no curl would be observable in response to nod factors. Furthermore, the roots respond to DMSO by growing no root hairs. It is not a good experiment, but we tried it. Experiments on the microtubules are being carried out by Dr Sharon Long.

E. Ralston: Fig. 4 shows the effect of 10 min exposure to Nod factor on actin distribution. But the pattern described is observed in only 25% of the root hairs showing actin labeling. Does this mean that only a small fraction of the root hairs respond to the stimulation, or that the pattern is transient and that different root hairs are not in phase? Would a higher fraction be observed to respond after longer exposure?

Authors: Many root hairs break during fixation and we cannot be sure that they are properly stained. We do not know the effect of longer exposure.

E. Ralston: In Fig. 3, the different panels show patterns of a wide range of brightness. Were all recorded with the same camera settings and were they all computer enhanced to the same degree?

Authors: Similar, but not exactly identical settings were used.

M. Terasaki: Is it possible that there is a Ca^{2+} gradient in the root tip, such as seen in growing pollen tubes and could that be the function of the accumulation of endoplasmic reticulum at the root tip?

Authors: There is no Ca^{2+} gradient [28]; the Ca^{2+} is high over the nuclear area and there are oscillations. We have seen a gradient occasionally from the tip with calcium green, again with most of the staining over the nucleus. So roots and pollen tubes differ.

Additional Reference

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